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THE USE OF TAMOXIFEN AS A BIOCHEMICAL MODULATOR IN ESTROGEN-RESPONSIVE HUMAN MAMMARY CARCINOMA

John Leonard Gwin, Jr.

1984

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THE USE OF TAMOXIFEN AS A BIOCHEMICAL MODULATOR IN ESTROGEN-RESPONSIVE HUMAN MAMMARY CARCINOMA

John Leonard Gwin, Jr.

A thesis submitted to the Yale University School of Medicine in partial fulfillment of the requirements for the degree of Doctor of Medicine.

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Abstract

Monolayer cloning studies were done to investigate the hormonal dependence of the 47-DN human mammary tumor cell line and the growth inhibition of sequential and combination antiestrogen-antimetabolite therapy. The antiestrogen Tamoxifen (TAM) was found to inhibit colony growth in a dose-dependent manner. The sensitivity of these tumor cells to TAM was found to be independent of the insulin concentration in the medium, while the presence of insulin definitely increased the sensitivity of the cells to the growth inhibitory effects of 5-fluorouracil (FUra).

The interactions of TAM with the antimetabolites methotrexate (MTX), FUra, and combined MTX-FUra (MF) were studied under conditions of sequential and combination treatment. For sequential experiments, TAM treatment is followed by washing out the drug and adding fresh medium containing estrogen before antimetabolite treatments. For combination therapy experiments, TAM administration precedes antimetabolite treatment, without intervening washing or addition of fresh media. TAM does not clearly interact with MTX synergistically, but at high doses of TAM, sequential TAM-MTX appears superior to combination treatment in growth inhibitory ability. On the other hand, high doses of TAM in combination with FUra are more cytotoxic than sequential TAM-FUra. Finally, TAM does enhance the cytotoxicity of MF synergistically if given in sequential fashion. The biochemical basis for this interaction may reside in the ability of TAM to effect cell cycle synchronization of the tumor cells.

Acknowledgements

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I. Introduction

A. Breast Cancer: Clinical Significance

Breast cancer, which affects approximately one out of eleven American women, represents a significant health problem in the United States (1). Surgery and radiation therapy are the dominant modes of therapy for the primary lesion; however, recurrence rates are high. Because disseminated disease is currently always fatal, improved methods of combination chemotherapy are needed (2).

The invasion of axillary lymph nodes by tumor cells at the time of mastectomy offers some prognostic insight into the development of recurrent disease. Twenty-one percent of patients with negative axillary nodes at surgery develop recurrent disease within 5 years, and 70% of patients with no axillary node involvement survive 10 years. Patients with 1 to 3 positive nodes develop recurrent disease within 5 years at a rate of 53%, while patients with more than 4 nodes involved have an 80% recurrence rate. Of all patients with axillary node involvement, less than 30% survive over 10 years (2). These figures certainly demonstrate the problem of recurrent disease and have led to attempts to reduce recurrence by the use of adjuvant chemotherapy in women with primary breast cancer and positive axillary nodes (3).

Currently, methotrexate (MTX) and 5-fluorouracil (FUra) are among the most commonly used drugs in the treatment of metastatic breast carcinoma. Individual response rates are on the order of 20 to 30%, and when used in combination with other agents, response rates of 50 to 60% may be achieved (5). *In vitro* studies have shown that synergistic

inhibition of the clonal growth of an estrogen-sensitive human breast tumor cell line results when MTX exposure precedes the administration of FUra (4). Preliminary clinical studies suggest that this sequence is highly effective in the treatment of advanced breast cancer (5). Tamoxifen (TAM) is a non-steroidal antiestrogen and has been used in the hormonal therapy of breast carcinomas containing estrogen receptors. In one study 22% of patients showed definite tumor regression in response to TAM therapy while 37% showed improvement with variable tumor regression (6). Investigation of the effects of combining TAM therapy with antimetabolite therapy such as sequential MTX-FUra may provide an improved mode of treatment of metastatic breast cancer.

B. Endocrinology of Breast Cancer

Estrogen receptors may be detected in 50 to 65% of all breast cancers (7). Studies of human mammary cell lines have generally failed to show an absolute dependence of the cells on estrogen for growth, but maximum growth is only obtained when appropriate concentrations of the hormone are present in the growth medium (8). Estrogen enters cells and binds to cytoplasmic estrogen receptors (ER). The resulting complex then enters the nucleus by a translocation process and binds to nuclear chromatin, by which it may direct the synthesis of specific ribonucleic acid (RNA) molecules (8,9). The loss of estrogen-receptor complex from the nucleus is referred to as processing. This processing step correlates with progesterone receptor (PgR) induction in the MCF-7 mammary carcinoma cell line, providing good evidence that stimulation of synthesis of PgR is one of the effects of estradiol on the cell (7). Other markers of estrogen action on cells include the induction of a secreted

glycoprotein in mammary carcinoma cell lines by estradiol (10).

The 47-DN human mammary carcinoma cell line is an appropriate system for the study of the endocrinology of breast cancer. The cell line, established from the pleural effusion of a patient with breast cancer, contains estrogen receptors, which are largely localized to the nucleus (11,12). The cells also show the induction of the aforementioned secreted glycoprotein upon exposure to estradiol (10). The growth of the 47-DN line is also sensitive to the effects of insulin (11), and this property aids in the usefulness of this system as a biological model of breast carcinoma in that Cohen and Hilf have reported that a certain group of carcinogen-induced mammary tumors in rats may be considered insulin dependent (13).

C. Tamoxifen

Harper and Walpole first showed the anti-estrogenic activity of TAM in evaluating a series of substituted triphenylethylenes (14). The structure of TAM is shown in Figure 1. This compound was found to

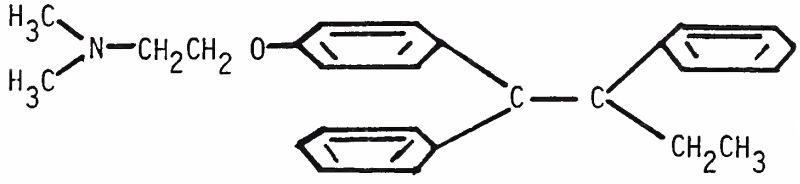

Fig. 1 Tamoxifen

counter the effects of estrogen on the vaginal epithelium and uteri of rats. It also prevents ovulation and terminates early pregnancy.

The drug was later used in the hormonal therapy of breast cancer as an alternative to the more drastic measures of oophorectomy and

adrenalectomy. Cole et al. showed definite tumor response in about 22% of patients treated with TAM (6). These patients received either 1 or 2 10mg tablets per day. Ward's early studies showed definite responses in 36% of patients treated with 10mg of TAM twice daily and partial responses in an additional 24%. In patients who were given 20mg TAM twice daily the definite response rate was 40%, and the partial rate 37%.

Numerous studies have been undertaken to determine the mode of the tumor growth inhibitory action of TAM. Studies of the MCF-7 cell line have shown that both TAM and nafoxidine (NAF) bind estrogen receptor and translocate them to the nucleus. While NAF results in no processing of the receptors, TAM leads to processing on the order of 30 to 50% of that seen with estradiol (8). Low doses of TAM (0.01 to 0.1μM) have an estrogenic effect, which may be measured by PgR induction. On the other hand higher doses (1μM) lead to the suppression of PgR levels below control levels and are accompanied by inhibition of cell growth and eventual cell death (8,16). Antiestrogens have also been shown to inhibit deoxyribonucleic acid (DNA) polymerase activity (17) in the MCF-7 cell line. The presence of 0.1μM 17-β-estradiol did not affect growth or DNA polymerase activity, and over a wide range of doses (0.0001 to 1000mM) estradiol had no significant effect on the activity of DNA polymerase. Both NAF and TAM reduced the activity of DNA polymerase when present in concentrations of 1μM; this reduction in activity could be reversed by the addition of estradiol. TAM was somewhat less effective than NAF in reducing DNA polymerase activity, and its effects could be reversed by lower doses of estradiol than for NAF inhibition.

Other workers have shown inhibition of macromolecular DNA synthesis by 0.1μM TAM in the MCF-7 line which could be reversed by estradiol administration (18).

Binding studies have yielded confusing information concerning the important binding sites of TAM in cells and their relationship to growth inhibitory effects. While the effects of TAM on cells and the ability of estradiol to prevent these effects suggests an estrogen receptor-mediated action for TAM, the work of some investigators has suggested that TAM is predominantly bound by a site distinct from the classical estrogen receptor in human breast cancer. Sutherland and Murphy used human mammary carcinoma biopsies to investigate competitive binding between TAM and estradiol (19). They found the presence of a saturable, high affinity TAM binding site in tumors which were shown to contain estrogen receptors but not in those which had no detectable receptor levels. While TAM is bound predominantly to its own site, it is able to inhibit the binding of 17-β-estradiol to its receptor with a relative binding affinity of 0.87 ± 0.35%. However, estradiol was unable to inhibit the binding of TAM to its binding site. The authors estimated that less than 5% of administered TAM is bound to the estrogen receptor. Sutherland *et al.* also reported the presence of this TAM binding site distinct from the estrogen receptor in the MCF-7 line and suggest that TAM is bound to both the ER and its own site (20).

Other studies only add to the complexity of the issue. The competitive binding studies of Nicholson *et al.* did show the ability of estradiol to inhibit TAM binding (21), but those investigators used lower ligand concentrations which may have led to the observation of greater TAM

binding to ER than seen in the studies of Sutherland and Murphy (19). Nevertheless, the estrogen receptor must have some relevance to the action of TAM. Butler et al. determined the effects of insulin on the sensitivity of MCF-7 to estrogen and antiestrogen (22). The addition of insulin to the growth medium decreases sensitivity to estrogen as measured by a decrease in production of plasminogen activator, a marker of estrogenic effects. The presence of insulin also decreased the sensitivity of the cells to TAM. The authors explain these findings by the ability of insulin to lower the number of estrogen binding sites from 62,000 ± 9,700 to 21,000 ± 4,700. The fact that TAM is more effective when there is an increased number of estrogen receptors present suggests that the growth inhibitory potential of TAM is due to some active inhibitory role of a TAM-ER complex, rather than mere competition between TAM and estradiol for ER binding sites.

TAM is able to inhibit the secreted glycoprotein induced by estrogen in the MCF-7 line (10), and this also supports some involvement of TAM in estrogen receptor interactions. Still, the presence of ER does not necessarily imply sensitivity to TAM. A cell line has been derived from MCF-7 which is resistant to the growth inhibitory effects of TAM but which still contains estrogen receptors (23). The effect of estradiol on cell growth is slightly weaker than in the TAM-sensitive MCF-7, but estrogen is still able to induce PgR in these cells, indicating the presence of an intact estrogen receptor mechanism. Clinical studies indicate that only 50 to 70% of patients with tumors containing estrogen receptors respond to antihormonal therapy (24), also indicating that the presence of ER is not an absolute predictor of TAM efficacy.

Human studies have shown an effect of TAM on estrogen receptors. Waseda et al. studied tumor specimens from patients who had been treated with TAM and found that while short term TAM administration could increase levels of nuclear estrogen receptor, long term administration led to a decrease in nuclear estrogen receptor content and a decrease in levels of PgR (25).

All of the above studies are not inconsistent with a model in which intracellular TAM binds to at least two sites. By binding to the classical estrogen receptor and preventing complete processing and also possibly binding to chromatin and actively inhibiting cellular processes, TAM clearly exerts an antiestrogenic effect. TAM may also bind to another specific site, which may or may not be involved in its actions on the cell. This site may in fact be analogous to the Type II sites reported in uterine cytosol (26). These sites may have a much lower binding affinity for estradiol and a higher affinity for TAM than the classical estrogen binding sites, and estradiol would thus be ineffective in inhibiting the binding of TAM. Clark et al. suggest that these Type II sites may serve to retain estrogen within cells, thus providing the classical sites with an estrogen-rich environment (26). This site could also bind TAM in large quantities, only a small but sufficient portion of which might be interacting with and altering the classical estrogen receptor mechanism.

Regardless of its mechanism of action, TAM is clearly able to inhibit DNA synthesis in MCF-7 (18). This effect may be reversed by the addition of estradiol to the medium. Recovery of the cells is accompanied by a rise in thymidine incorporation rates to levels above control values

during the early phase of estradiol rescue of the cells. This observation suggests that a greater proportion of the cells are entering the DNA synthetic phase of the cell cycle, and so TAM has effected a synchronization of the tumor cell population. Therefore, TAM may have some role as a synchronizing agent in possible combination chemotherapy regimens for breast cancer.

Pharmacokinetic studies suggest that single daily doses of 20mg/m^2 of TAM are sufficient to maintain blood levels in the therapeutic range ($\geq$150 mg/ml) after 8 weeks of treatment (2,7). This blood level is equivalent to 0.55μM. Peak concentrations are found in blood 4 to 7 hours after oral administration. TAM concentration decreases in a biphasic fashion with an initial half life of 7 to 14 hrs, followed by a terminal half life longer than 7 days. TAM is metabolized by hydroxylation and conjugation; most metabolites are excreted in the stool (2,8). Side effects of TAM are rare and usually tolerable including hot flashes, nausea, vomiting, tumor pain and headaches most commonly (6,15).

D. Methotrexate

Methotrexate (MTX) exerts antitumor action by virtue of its antimetabolic effects as an analogue of folic acid (28). MTX produced the first remissions in leukemia and is useful in choriocarcinoma, acute lymphocytic leukemia, and solid tumors of the breast, ovary, head and neck, and lung. The structure of MTX is given in Figure 2.

MTX enters cells by a carrier-mediated process and exerts its cytocidal effect via tight binding and inhibition of the enzyme dihydrofolate

reductase. Enzyme blockade is only achieved if free intracellular MTX is in excess to the target enzyme. This inhibition prevents regeneration of reduced folic acids, and depletion of tetrahydrofolic acid pools results as reduced folates are oxidized in the reaction catalyzed by thymidylate synthetase (Figure 3).

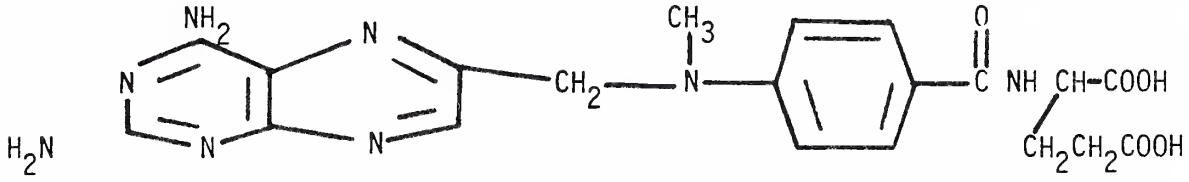

Figure 2. Methotrexate

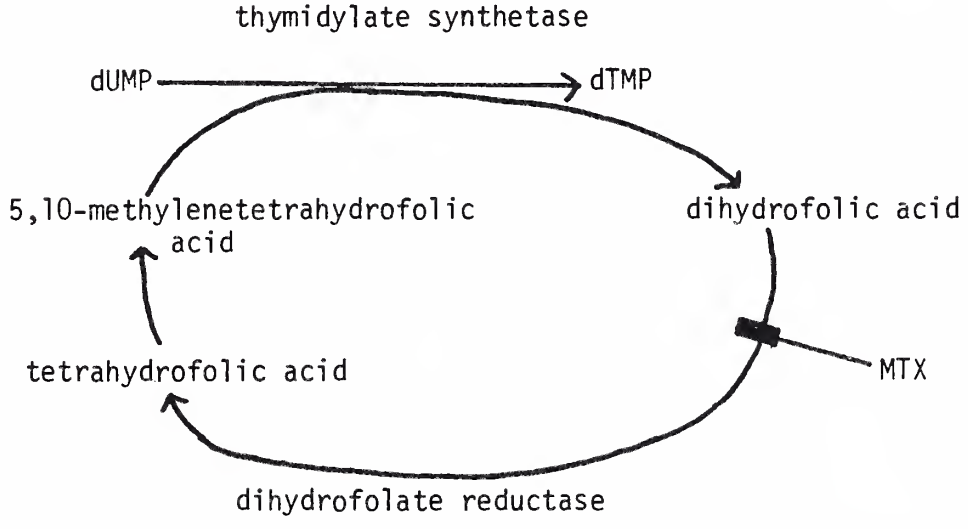

Figure 3. The enzymatic conversion of deoxyuridylate to deoxythymidylate.

This can have effects on a number of biosynthetic pathways in nucleic acid biosynthesis, as shown in Figure 4. The purine biosynthetic pathway is blocked at the step involving conversion of glycinamide phosphoribose to formylglycinamide phorphoribose. The subsequent accumulation of phosphoribosyl pyrophosphate (PRPP) can modulate the pyrimidine pathway (29).

Cytocidal effects of MTX are dependent on the cells' entering the DNA synthetic phase of the cell cycle, for pyrimidine biosynthesis must be occurring to deplete tetrahydrofolate pools. In order to achieve high intracellular concentrations of MTX, high doses are occasionally given to patients. Because of potential serious toxic effects of these doses of MTX, the patients are then "rescued" by the administration of leucovorin, or 5-formyltetrahydrofolate. This serves to replenish depleted reduced folate pools and restore normal cell metabolism.

The toxicity of MTX is self-limiting. Because of the wide uses of folate in cell metabolism, MTX inhibits RNA and protein synthetic mechanisms, thus slowing the entry of cells into the DNA synthetic phase. The cells therefore become less sensitive to the lethal effects of MTX. Resistance to MTX is also found in some cells and may occur by one of three mechanisms. Impaired transport of MTX into cells leads to the inability to achieve the necessary high intracellular concentration of MTX. A second mechanism for resistance involves the possession of a form of the enzyme dihydrofolate reductase with a reduced affinity for MTX. A third means is from increased levels of dihydrofolate reductase which may be achieved by enzyme induction.

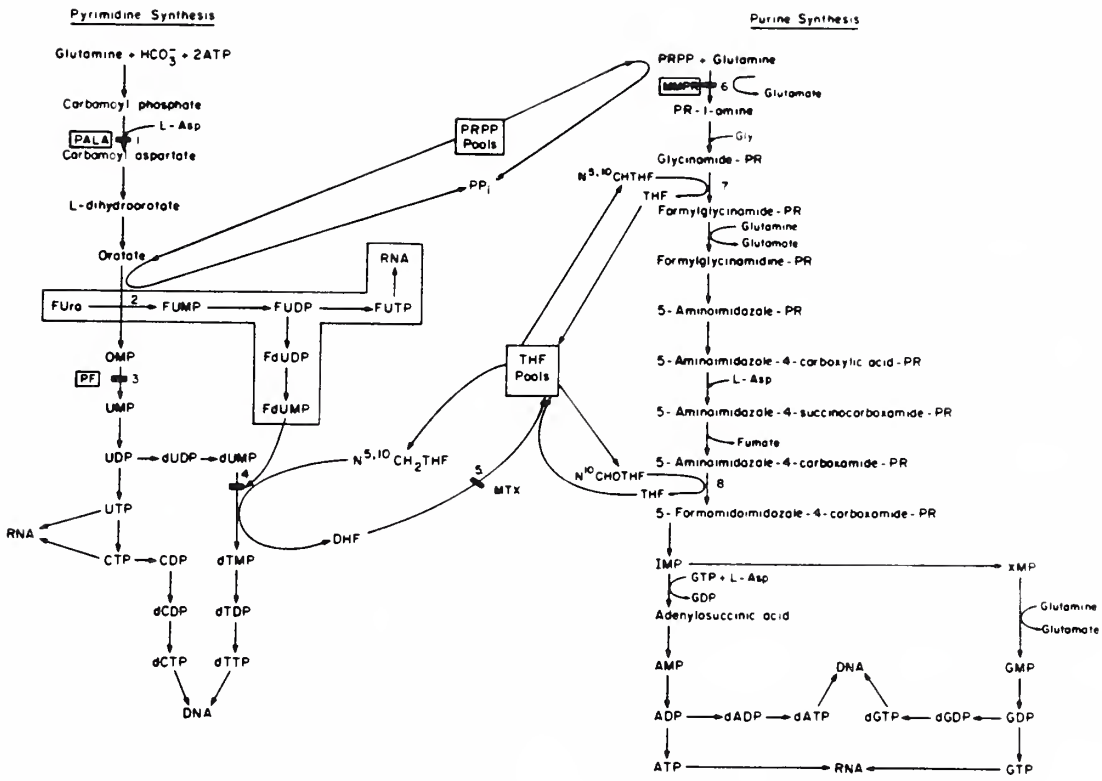

Figure 4. Nucleic acid biosynthetic pathways.

While highest response rates to MTX have been observed in rapidly growing tumors, MTX also has beneficial effects in a number of slowly growing solid neoplasms. MTX also has been used in the adjuvant treatment of cancer. The rationale for this is that after surgical removal of the primary lesion, MTX is highly active against small tumor foci with a high growth fraction. Furthermore, the patients are more capable of tolerating drug-related toxicity when they are least debilitated by the tumor.

(29). As was mentioned above, individual response rates of patients with advanced breast cancer are 20 to 30% (5). MTX adversely affects the intestinal tract, bone marrow, kidneys and liver; intrathecal administration may cause neurotoxicity.

E. 5-Fluorouracil

5-Fluorouracil (FUra) (Figure 5) must be metabolized by cells for cytotoxic effects to be realized (31). Anabolic mechanisms (Figure 6) may convert FUra into fluorouridine (FUrd), fluorodeoxyuridine (FdUrd), or fluorouridylate (FUMP). Fluorodeoxyuridylate (FdUMP) may be formed either by phosphorylation of FdUrd or from FUMP via FUDP and FdUDP. FdUMP represents one of the major cytotoxic metabolite of FUra, for it inhibits the enzyme thymidylate synthetase in the presence of 5,10-methylenetetrahydrofolic acid. The three species are necessary for a stable ternary inhibitory complex. This inhibition prevents the formation of deoxythymidylate and thus DNA, so that this means of FUra toxicity is dependent on the cells entering DNA synthesis, as for MTX. Cell death has also been associated with chromosomal breaks (32).

There exists another pathway for FUra cytotoxicity however, and this involves the conversion of FUMP to FUDP and then FUTP and its incorporation into various RNA species. The product of such "fraudalent" RNA molecules may lead to miscoding during translation of messenger RNA (mRNA) into protein or to alteration in normal post-transcriptional processing and maturation of mRNA. The relative importance of these two major mechanisms of cytotoxicity of FUra remains a point of controversy. The contribution of each probably depends heavily on the metabolic states of the cells involved and may be manipulated pharmacologically.

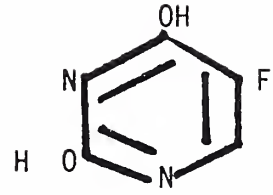

Figure 5. 5-Fluorouracil.

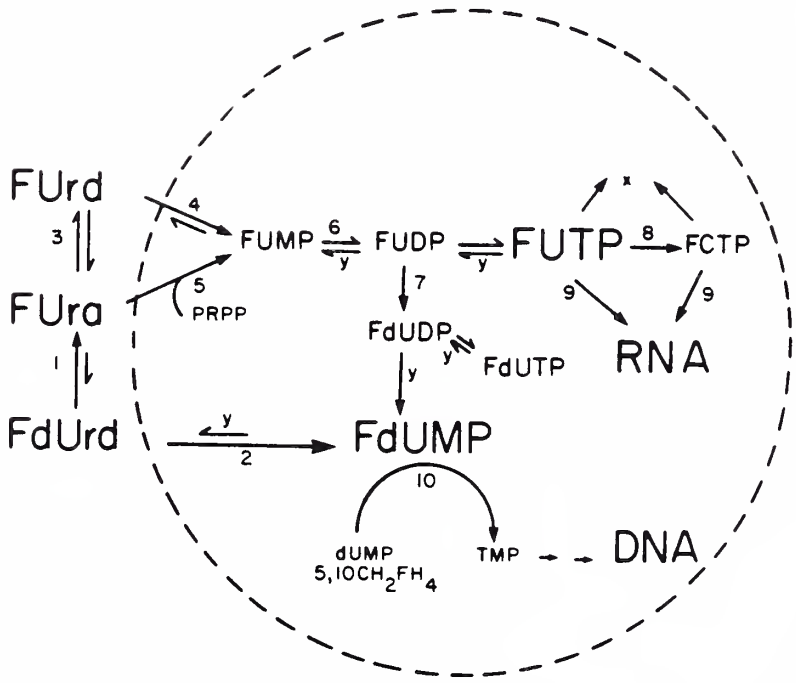

Figure 6. Pathways of FUra metabolism

Key: 1. Thymidine, deoxyuridine phosphorylase
2. Thymidine kinase
3. Uridine phosphorylase
4. Uridine kinase
5. Phosphoribosyltransferase
6. Uridylate kinase
7. UDP reductase
8. CTP synthetase
9. RNA polymerase
10. Thymidylate synthetase

x Degradation products
y Phosphatases

Because gastrointestinal absorption of the drug is unpredictable and incomplete, FUra is usually administered intravenously. The clinical uses of FUra include palliative treatment of carcinomas of the breast, gastrointestinal tract, liver, ovary, cervix, urinary bladder, prostate, pancreas and oropharyngeal areas. FUra is cytotoxic to bone marrow and gastrointestinal epithelium, so that clinical toxicity is manifested by anorexia, nausea, vomiting, diarrhea, stomatitis, and leukopenia. Resistance to FUra may develop and may be due to a decrease in uridine kinase activity, a decrease in phosphoribosyltransferase activity, or the presence of a resistant form of thymidylate synthetase (28,31,32).

F. Methotrexate-5-Fluorouracil Interactions

The interactions of MTX and FUra have been studied extensively. If FUra exerts its primary cytocidal effect via its inhibition of thymidylate synthetase, then the addition of MTX should antagonize its effects. The MTX-induced depletion of tetrahydrofolic acid pools theoretically prevents the formation of the ternary complex of thymidylate synthetase, FdUMP, and 5,10-methylenetetrahydrofolate. It appears that MTX, as an analogue of folic acid, may participate in the forming of such a ternary complex, but such a complex does not seem to be found to any great extent in experimental systems (29). Experimentally, drug synergism is observed when MTX administration precedes FUra (4,29,33). Since MTX probably antagonizes the FdUMP-mediated cytotoxic effect of FUra, a search for another mechanism for this synergism was undertaken.

FUra may also exert a toxic effect through its incorporation into RNA,and MTX enhancement of this incorporation may explain the syner-

gism. Cadman et al. showed that MTX treatment of the murine leukemia cells L1210 leads to increased PRPP pools which can cause increased formation of intracellular FUra nucleotides. PRPP is used in purine biosynthesis, so that MTX-mediated inhibition of the purine biosynthetic pathway leads to a decreased utilization of PRPP and a subsequent accumulation of PRPP within the cell. These PRPP molecules may serve to form FUra nucleotides, including both FUTP and FdUMP. Because MTX antagonizes the FdUMP-mediated cytotoxic effect, the observed synergism between MTX and FUra is likely due to the increased formation of FUTP and its incorporation into RNA. The mechanisms for cytotoxicity as a result of this fraudulant RNA were discussed above.

In the human breast carcinoma cell line, 47-DN, maximal cytotoxicity is present when FUra is administered in the last 6 hours of a 24-hour MTX treatment (4). MF has also demonstrated potential benefit in the clinical treatment of advanced breast cancer (5).

G. Antiestrogen-Antimetabolite Interactions

Further enhancement of the antitumor effects in breast cancer of these antimetabolites used either singly or in combination may be achieved via combining them with hormonal therapy, particularly TAM. Several clinical studies have been undertaken to determine the efficacy of combining the modalities of hormonal and chemotherapy of breast cancer. One of these compared the use of either TAM, L-phenylalanine mustard, and FUra or L-phenylalanine mustard and FUra alone in the adjuvant chemotherapy of patients with one or more positive axillary nodes (3). Patients were given TAM twice daily for the 2 years of the protocol. L-phenylalanine mustard and FUra were administered daily on the first 5

days of a 6-week cycle. Overall, there was significantly increased disease-free survival of those patients treated with TAM plus chemotherapy. The main group which benefited were the patients >49 years old with high ER levels. In patients ≤49 years old, the only group receiving benefit from TAM were those with 4 or more positive nodes and high ER levels. The administration of TAM produced no additional toxicity other than an increase in the incidence of hot flashes.

Another study reporting benefit from the addition of TAM to chemotherapeutic regimens in the adjuvant treatment of breast cancer is that of Hubay et al. (34). This group compared the use of cyclophosphamide (Cytoxan), MTX, and FUra (CMF), and CMF plus TAM (CMFT). Treatment was given according to 28-day cycles, in which cytoxan was administered on days 1 through 14, while MTX and FUra were given on days 1 and 8. Patients received TAM daily for one year. Patient response depended upon ER status; CMFT treatment was significantly more effective in delaying recurrence than CMF alone in patients with ER-containing tumors, regardless of menopausal status.

One group has reported preliminary results on the use of TAM and chemotherapy in the treatment of advanced breast cancer (35). The drug regimen involved TAM treatment for 10 days, followed by estrogen therapy for 4 days, on the last day of which patients received MTX-FU treatment. The rationale for this study involves the concept of estrogen rescue and stimulation of ER-containing breast cancer cells, which should be sensitive to the effects of sequential MTX-FU. Only 9 patients have been evaluated, and 7 responded to the therapy. In fact 5 attained a complete remission. While this study obviously can only offer preliminary results, the conclusion to be obtained from all

these clinical studies is that there is some rational basis for combining hormonal and chemotherapeutic regimens in the treatment of breast cancer.

How might TAM interact with the drugs discussed above? In a "combination" regimen, TAM could be given and another agent or agents could be given either simultaneously or at some later time when TAM was still present. "Sequential" therapy would involve TAM administration followed by an "estrogen rescue" (18,35), after which the antimetabolites would be given.

A priori, the combination of TAM and an S-phase specific agent such as MTX might be expected to be antagonistic, since TAM inhibits DNA synthesis (18). Furthermore, the synergism of MF is dependent on DNA synthesis and TAM-dependent inhibition of this process might decrease the cytotoxicity of the antimetabolites. The effects of combining TAM and FUra are certainly less predictable, because FUra's cytotoxic actions involve at least 2 mechanisms, only one of which is dependent on cells' synthesizing DNA.

The sequential therapy takes advantage of the fact that TAM followed by estrogen rescue may result in some degree of cell cycle synchronization of the tumor cells (18). Cell cycle synchronization results in an increased proportion of cells entering S-phase following rescue. An increased proportion of cells in S-phase does increase cell killing by S-phase specific agents (36), so that TAM-induced synchronization of breast cancer cells may increase MTX and MTX-FUra cytotoxicity. Synchronization of cells would not necessarily increase the cytotoxicity of FUra, because its effects are not limited to cells which are actively synthesizing DNA.

Cytotoxicity of MTX has been shown to increase with proliferative rate of tumors (37), and several investigators have shown than tumors with high growth fractions (which may occur during rebound from a cell cycle block) respond to cycle-specific therapy better than those with a low growth fraction (38,39,40,41). Steel has identified one problem in the application of cell cycle synchronization to therapeutic use, and that is that tumor synchronization and increased tumor cell kill will be accompanied by normal host tissue synchronization and increased normal cell kill and increased host toxicity, with no subsequent improvement in therapeutic index (42). This problem may be circumvented with the use of an agent such as TAM, whose synchronizing effects should only be observed in cells containing estrogen receptors. Host toxicity should not then be increased to any great extent.

H. Objectives

In vitro studies may answer many of the questions concerning TAM and antimetabolite interactions. The use of the human breast cancer cell line, 47-DN, provides a suitable experimental system for studying this problem. This work proposes to evaluate the growth inhibitory effects of TAM alone in the 47-DN cell line and also the hormonal dependence of TAM cytotoxicity. The hormonal dependence of FUra effects on the cells is also examined. Finally,the interactions of TAM and MTX,FUra,and MF in both combination and sequential fashion are evaluated according to ability to inhibit colony growth of 47-DN.

II. Materials and Methods

A. Cells

The 47-DN breast tumor cell line is a well-characterized, continuously growing monolayer cell line (11), which has a doubling time of 26 hours under our culture conditions. Cells were maintained in Roswell Park Memorial Institute Medium 1640 (Grand Island Biological Co. (GIBCO), Grand Island, NY) supplemented with 10% of a 1:1 mixture of fetal bovine serum and newborn calf serum (GIBCO). Stocks were grown in media containing 0.2 IU/ml insulin (Eli Lilly Co., Indianapolis, IN) and 1nM estradiol (Sigma Chemical Co., St. Louis, MO). Medium changes made for certain experiments are described under "Results." The estradiol content of serum was measured and found to be less than 100pM. Stocks were passaged weekly using a trypsin (0.05%)-EDTA (0.02%) solution and vigorous pipetting to obtain a suspension of >90% single cells. Cells were grown in 75 cm^2 sterile plastic culture flasks (Costar Data Packaging, Cambridge, MA) with 25ml medium in 5% CO_2 incubators at 37°C. All cell counts were performed on a Coulter Model ZBI Counter (Hialeah, FL).

B. Drugs

TAM, obtained from ICI Americas, Inc. (Wilmington, DL), was dissolved in absolute ethanol and kept in a 0.01M stock solution. All dilutions were made with ethanol, and control flasks in TAM experiments received a volume of ethanol equivalent to that received by TAM treated flasks. Solutions of MTX (Sigma), were made in sterile phosphate buffered saline (PBS) from 0.001 M stock solutions. FUra (Sigma) was also dissolved in PBS to give a stock solution of 0.01 M, from which PBS dilutions were made.

C. Monolayer Colony Growth Experiments

Stock cultures were washed with PBS, and cells were removed by trypsinization and vigorous pipetting. 30,000 to 40,000 cells were seeded into each flask with 25ml of medium and grown in 5% CO_2 incubators at 37°C. In some experiments involving multiple drug treatments, >40,000 cells were seeded in each flask; final colony counts were corrected accordingly. On the 3rd to 4th day after seeding, when colonies consisted of 4 to 32 cells, drug treatments were begun. These small colonies are representative of the micrometastases thought to be present in human malignancies. Up to 90% of cells can be killed without a change in the number of colonies which grow, so colony growth inhibition represents quite significant cell kill (43). Volumes of either 25µl or 250µl of drug solution were added to flasks to obtain appropriate final concentrations in the medium. Drug treatments were terminated by pouring off of medium, washing with PBS, and adding fresh medium. TAM was given for variable times, MTX for 24 hours, and FUra for 6 hours. MF treatments involved administering FUra for the last 6 hours of a 24-hour MTX exposure. In some experiments, as noted in "Results," just before drug treatment, the medium was changed to RPMI 1640 + 0.2 IU/ml insulin + 1.0 nM estradiol +10% dialyzed fetal bovine serum. After all drug treatments were terminated, cells were allowed to grow until control flasks reached a density of approximately 1000 cells per 2000 mm^2. At this time, usually about 10 days after seeding, the medium was removed from all flasks and cells were washed with PBS and stained with a methanol-crystal violet (2.5%) solution. Colonies were enumerated using an

automated colony counter (Biotran II; New Brunswick Scientific Co., Inc., Edison, NJ). The sensitivity of the counter was set to obtain maximum counts from control flasks in counting fields of 2000 mm^2. Three fields were counted per flask, and with at least 2 flasks per experimental condition, at least 6 data points were obtained from which a standard deviation could be calculated. Comparison of manual counting using an inverted microscope and colony counts from the counter showed that automated counts were approximately one half of manual counts. This means that the true cloning efficiency of the system is approximately 20%, while the effective efficiency is approximately 10%, assuming a count of 1000 colonies per 2000 mm^2 field of a 75 cm^2 flask seeded with 40,000 cells.

D. Data Analysis

For each growth condition, at least 2 simultaneous experiments were done, so that 6 data points were obtained from which means and standard deviations could be calculated. In the FUra sensitivity studies, an analysis of variance between the means obtained from two experiments is performed. In the combination drug studies, many more experiments were done, and because of the great deal of variability among experiments, an ICE value (index of combined treatment effect) is calculated. Because each experiment done with a set of flasks seeded at one time involves both single and multiple drug treatments, it is possible to determine if the observed growth is greater or less than that expected if additive effects were occurring. For example, for an experiment with MTX, FUra and MF, the ICE for MF

is equivalent to % control growth for MF/ (% control growth for MTX) (% control growth for FUra). An ICE>1 implies that the drugs are interacting antagonistically, while ICE values <1 indicate drug synergism. An ICE =1 indicates purely additive effects. Mean ICE values ± S.D. are calculated from at least 6 data points and a two-tailed t test is performed to determine significant difference from 1.0. In an experiment performed in dialyzed serum, ratios of % control growth after multiplied drug treatments to % control growth in TAM only are calculated, and sequential and combination type schedules are compared using a two-tailed t test.

III. Results

A. Tamoxifen Sensitivity of 47-DN

To determine the ability of TAM to inhibit colony growth of 47-DN, cells were treated with concentrations of TAM ranging from 0.01μM to 10μM at exposure times ranging from 1 hour to continuous exposure. Figure 7 shows the clonal growth of these treated cells. In this experiment the medium contained 2 IU insulin/ml. Drug or ethanol treatments involved the addition of 25μl to each flask. Growth inhibition is clearly seen beginning with a 24-hour exposure to 10μM TAM. 10μM TAM for 48 hours leads to approximately 30% growth, while 0.01μM TAM has little effect on 47-DN.

Cells which were growing in RPMI 1640 + 1nM estradiol and several insulin concentrations were exposed to various doses of TAM for 48 hours and colony growth determined. Figure 8 shows that there is no real difference in sensitivity of the various groups of cells. 10μM TAM produces from 50 to 60% inhibitionof colony growth of cells grown in all media.

B. Sensitivity of 47-DN to FUra

A dose-response relationship for FUra treatment of 47-DN was established by treating cells grown in 4 different types of media. All were grown in RPMI 1640, with either one or both or neither of 1nM estradiol or 2 IU/ml insulin. Figure 9 shows that a 6-hour exposure to 5μM FUra produces greater inhibition of growth when insulin is present than when no exogenous insulin is added to the medium. This effect is

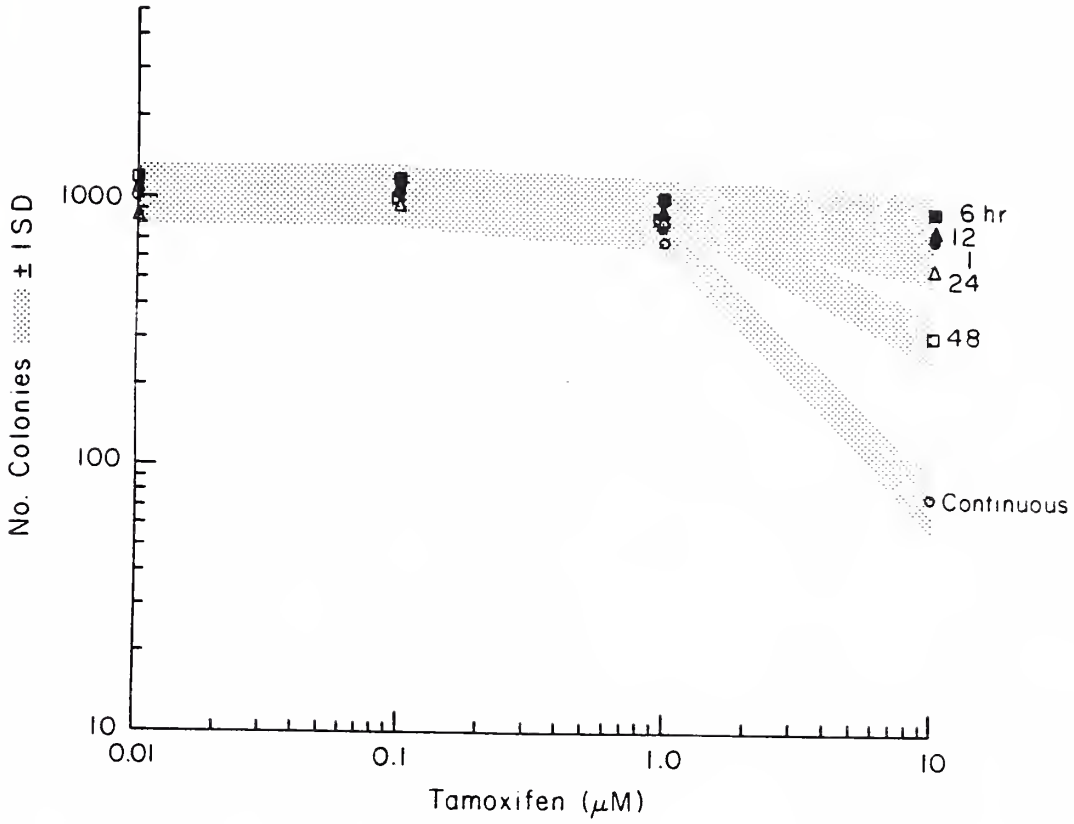

Figure 7. Clonal growth of Tamoxifen treated 47-DN.

Cells were exposed to varying concentrations of TAM for varying times beginning on the 3rd to 4th day after seeding. Growth was assayed as described in methods and expressed as mean number of colonies per 2000 mm^2 area ± 1 standard deviation. Each condition represents 6 data points obtained from two separate, simultaneous experiments.

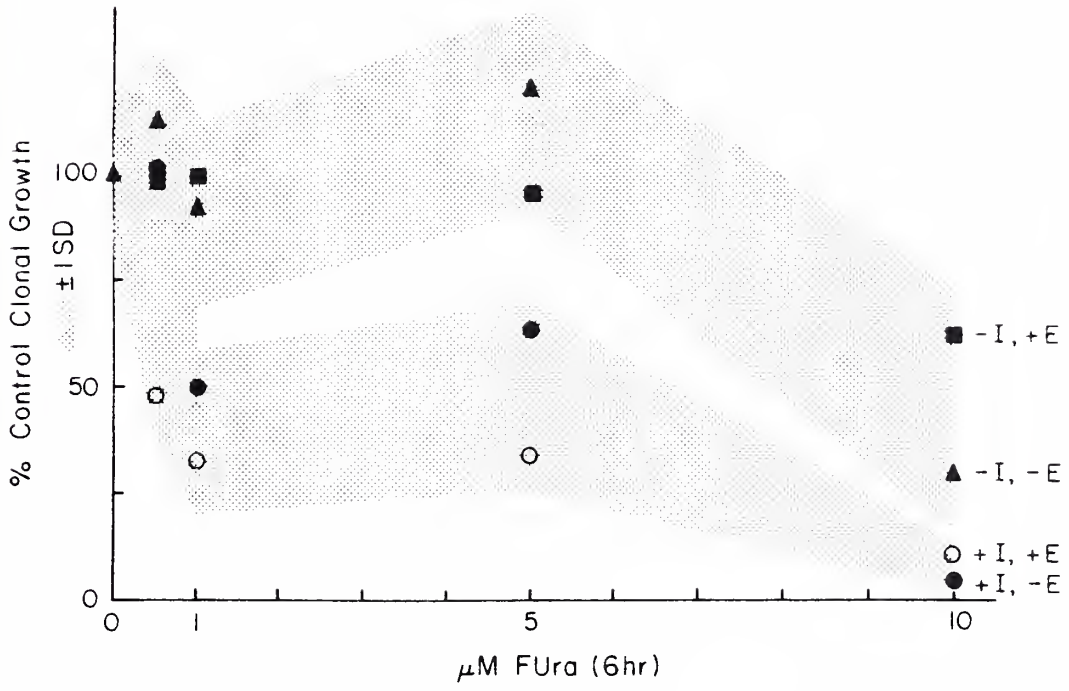

Figure 9. 47-DN sensitivity of FUra ± Insulin (I), ± Estradiol (E).

Cells were grown in RPMI 1640 + 1 nM estradiol + 4 different concentrations of insulin (0, 0.02, 0.2, 2 IU/ml) and exposed to varying concentrations of 48 hour TAM. Growth is presented as mean % control growth ± range of mean value for two separate experiments, each involving duplicate flasks.

Table 1

INSULIN DEPENDENT SENSITIVITY OF 47-DN TO FUra

μM FUra (6hr)	Mean % Clonal Growth -insulin	+ insulin	p*
0.5	100	84	>.10
1.0	97	70	>.10
5.0	93	51	<.05
10.0	40	25	>.10

* one-way analysis of variance

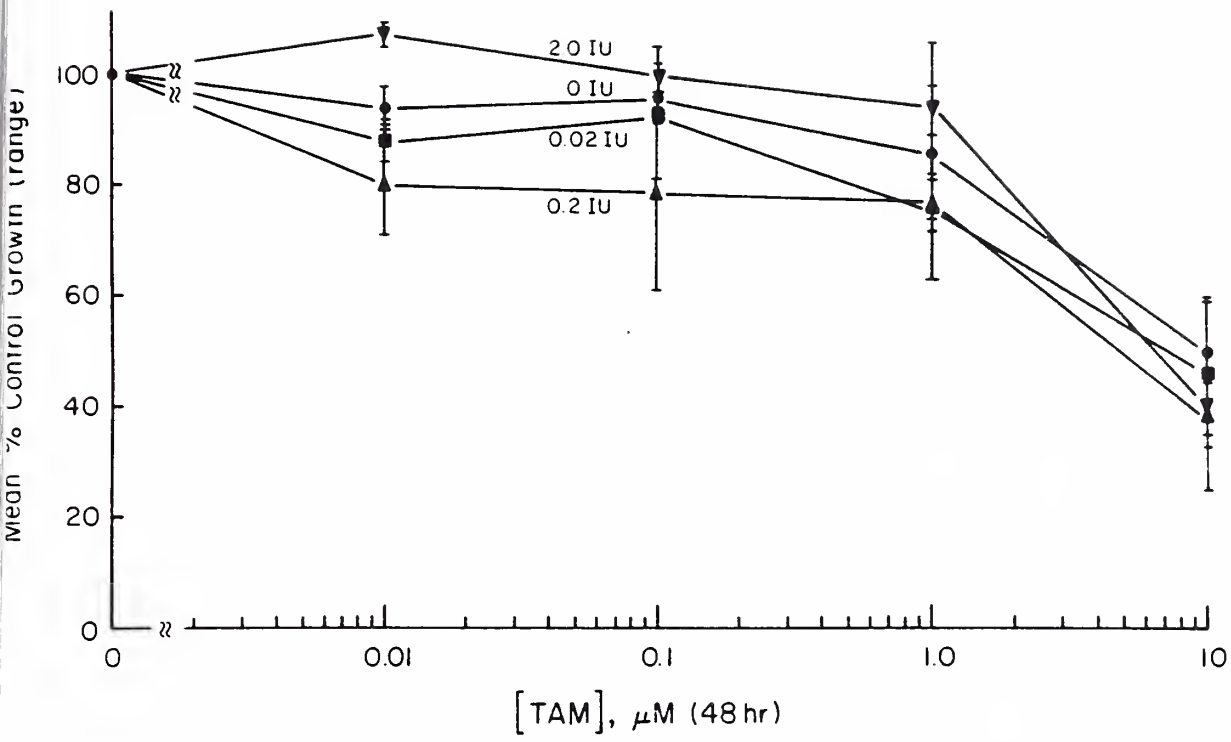

Figure8 . Effect of Insulin on 47-DN sensitivity to Tamoxifen.

Cells were grown in RPMI 1640 sith varying combinations of lnM estradiol and 2 IU/ml insulin and exposed to 6 hour FUra. Clonal growth is expressed as percentage of control growth ± S.D. determined from 6 data points from duplicate, simultaneous experiments

independent of estradiol content of the medium. Table 1 shows the mean results of two such experiments, one of which is presented graphically in Figure 9. The other experiment was identical except that 0.2 IU/ml insulin was used. Clearly, the presence of insulin significantly increases the sensitivity of 47-DN to the growth inhibitory effect of FUra.

C. TAM-Antimetabolite Interactions

A number of experiments were undertaken to examine the interactions of TAM, MTX, FUra, and MF. Doses of 0.5μM and 5μM were used for MTX and FUra, respectively, on the basis of previous work (44), and cells were exposed for 48 hours to either 0.01μM TAM or 10μM TAM. In "combination" experiments, MTX is given during the last 24 hours of the 48 - hour TAM exposure and FUra is given in the last 6 hours. In "sequential" experiments TAM is administered for 48 hours, after which the drug is washed out, medium replaced and antimetabolite treatments begun immediately in the case of MTX, or 18 hours later, in the case of FUra. Table 2 presents the results of a number of such experiments. Each experiment involved duplicate flasks for each condition and the numbers presented here represent the mean of at least two such experiments.

These results confirm the synergism between MTX and FUra when MTX precedes FUra. Combination of TAM and MTX is antagonistic at high and low doses, while sequential TAM-MTX is additive. The combination of 10μM TAM and FUra shows a synergistic interaction while sequential treatment is additive. When 0.01μM TAM is used, both sequential and combination treatment are antagonistic. Finally, the results show that sequential TAM-MF is a synergistic combination

high dose TAM, but additive with low dose TAM; simultaneous use of either high or low dose TAM with MF is antagonistic.

Another experiment, performed using dialyzed serum, sought to compare directly the interaction of 10μM TAM with MTX,FUra and MF. For combined treatments, cells were seeded at 200,000 per flask and corrections were made to calculate % control growth. Furthermore, because treatments were begun at various times after seeding, each combination had its own ethanol and TAM controls. However, identical antimetabolite treatments are performed at the same time. The results are presented in Table 3 (these results were also used in determining the values for Table 2). This experiment confirms the previous results in showing that sequential TAM-MF inhibits colony growth of 47-DN to a greater degree than combination TAM-MF. It also shows the superiority of combination TAM-FUra over sequential TAM-FUra in growth inhibition. These results finally show a significant difference between sequential TAM-MTX and the simultaneous combination of TAM-MTX.

Table 2

TAM-Antimetabolite Interactions

	Mean % Growth ± S.D.	Mean ICE ± S.D.	Interaction, p value*
	51.2 ± 22		
	130 ± 58		
	71.0 ± 35		
	44.0 ± 15		
d 5	14.5 ± 13	0.48 ± 0.4	synergistic, $p<0.01$
MTX 0.5	35.7 ± 14	1.73 ± 1	antagonistic, $p<0.05$
+ MTX 0.5	109 ± 50	2.33 ± 2	antagonistic, $p<0.05$
X 0.5	32.8 ± 30	1.08 ± 0.4	additive, $p>0.10$
MTX 0.5	110 ± 11	1.21 ± 0.1	additive, $p>0.10$
FUra 5	16.5 ± 7	0.49 ± 11	synergistic, $p<0.01$
+ FUra	82.4 ± 31	1.30 ± 0.3	antagonistic, $p<0.01$
ra 5	27.2 ± 8	1.01 ± 0.3	additive, $p>0.10$
FUra 5	41.4 ± 6	1.32 ± 0.2	antagonistic, $p<0.05$
MF	18.6 ± 10	11.2 ± 11	antagonistic, $p<0.01$
+ MF	18.2 ± 6	5.68 ± 5	antagonistic, $p<0.01$
	7.1 ± 3	0.64 ± 0.3	synergistic, $p<0.05$
-MF	25.4 ± 10	0.79 ± 0.3	additive, $p>0.10$

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ıtion

-FUra (see text)

*two-tailed **t** test for the null hypothesis, ICE = 1

Table 3

TAM-Antimetabolite Interactions in Dialyzed Serum

	Ratio % Growth in Drug treated/% Control growth[∓]		p*
	Combination	Sequential	
d MTX 0.5	0.73 ± 0.08	0.31 ± 0.15	p<0.01
d FUra 5	0.28 ± 0.005	0.68 ± 0.19	p<0.01
d MF	0.60 ± 0.07	0.25 ± 0.09	p<0.01

atments consist of TAM-Antimetabolite treatments in either combination or al fashion; control growth is growth in TAM alone.

ed t test for the null hypothesis: combination and sequential treatments equal growth.

of TAM before MTX and before sequential MTX (M) and FUra (F) was superior to of TAM simultaneously with these drugs. TAM, however, resulted in the greatest of cells exposed to FUra when it was added with the FUra and not when it was ·ior to FUra.

V. Discussion

The dose response relationship obtained for TAM inhibition of clonal growth of the 47-DN human mammary tumor cell line indicates that these cells are sensitive to the growth inhibitory effects of this antiestrogen. The cells do contain estrogen receptors (11), but this does not necessarily imply TAM sensitivity (23). Estrogen receptor measurements have been made on this cell line under control conditions and after TAM treatment (10μM, 72 hours). The addition of TAM reduces cytoplasmic ER 5-fold and PgR 4-fold (46). These results indicate that TAM is altering the normal course of events in estrogen receptor processing and suggest that the growth inhibitory properties of TAM are mediated through the ER. Clinical studies have shown that TAM blood levels of 0.55μM are therapeutically effective (27), and in the 47-DN line continuous exposure to this concentration of TAM does show some inhibition of colony growth. Blood levels greater than 1μM are achievable clinically, and the in vitro data suggest that these levels would have greater ability to inhibit breast tumor growth.

The apparent independence of the TAM sensitivity of 47-DN to insulin present in the medium shows the insulin-estrogen relationship of 47-DN to be different from that of MCF-7. Butler (22) has shown that the presence of insulin lowered ER and TAM sensitivity in MCF-7, but the presence of insulin had no effect on the sensitivity of 47-DN to TAM. Studies of these hormonal relationships in other mammary tumor systems have indicated several mechanisms depending on the tumor's response to insulin. In DMBA-induced mammary tumors which regress when the rats

are made diabetic, replacement of insulin leads to tumor growth and increased estrogen binding capacity (47). Insulin independent tumors behave in a manner similar to MCF-7, for the absence of insulin leads to an increased ER content. Furthermore, there exist some tumors which remain static under insulin deprivation, and the ER content is not affected by insulin addition or deprivation (48). The 47-DN cell line seems to fit most closely with this last model.

While insulin does not appreciably affect the sensitivity of 47-DN to TAM, it has a marked influence on the cytotoxicity of FUra. The mechanism of insulin's enhancement of FUra cytotoxicity may possibly be understood by a consideration of insulin's modulation of biochemical pathways within the cell. In a previously mentioned study (13) involving insulin-dependent DMBA-induced mammary tumors in diabetic and intact rats, insulin deprivation leads to tumor regression accompanied by decreases in RNA levels, increases in DNA levels, and decreased activities of glycolytic enzymes and enzymes of the hexose-monophosphate pathway. While the TAM studies of 47-DN indicate that the cells may not be dependent on insulin, they nevertheless may respond biochemically to the presence of insulin. Insulin definitely stimulates the hexose-monophosphate pathway in liver cells and adipocytes (49). The hexose-monophosphate pathway serves to generate reducing power in the form of NADPH, to convert hexoses to pentoses, and to complete oxidative degradation of pentoses (50). The second function is important for this discussion, for the pentose formed, D-ribose-5-phosphate, is required in the synthesis of nucleic acids. PRPP is formed from D-ribose 5-phosphate in one

step at the expense of ATP (49), and the role of PRPP in the toxicity of FUra is discussed above. The presence of insulin is therefore necessary for maximum formation of FUra nucleotides which may serve to inhibit DNA synthesis or become incorporated into RNA. In the absence of insulin, cells may have lower PRPP pools available for the metabolism of FUra which is necessary for its ability to inhibit growth of cells.

The interaction of TAM and MTX in inhibiting the growth of 47-DN is as expected. Pretreatment with TAM and rescue with fresh medium followed by MTX should be more cytotoxic than TAM pretreatment followed by MTX with no rescue. TAM inhibits DNA synthesis (17) and since MTX's lethal effects are dependent upon the cells' synthesizing DNA (28), the two drugs should interact in an antagonistic manner if given together. Treatment and rescue might be expected to cause a certain degree of synchronization of the tumor cells, which should enhance MTX toxicity (42). In fact flow cytometric studies show that TAM can produce a G_1 or G_1-S block at growth inhibitory concentrations (46). Sequential treatment with 10μM TAM and MTX was superior to combination treatment, but true synergism was not seen with either low or high dose TAM.

The interactions of TAM and FUra are consistent with the work of Benz and Cadman, in that 10μM TAM in combination with FUra is synergistic and superior to sequential therapy. The interaction of TAM and FUra may thus be highly dependent on TAM concentration because synergism is only observed with high doses and the low dose of TAM interacts antagonistically with FUra. If sequencing does result in synchronization of the cycling of the tumor cells, we would

not expect it necessarily to enhance the cytotoxicity of FUra. The lethal effects of FUra are not dependent on DNA synthesis (29), and synchronization with other agents has been shown not to affect the cytotoxicity of FUra (42).

At least two mechanisms may be considered in an attempt to explain the interaction between TAM and FUra. Two groups of investigators have reported that RNA molecules are able to inhibit the binding of ER complexes to DNA (51,52) and suggest that steroid receptor complexes may participate in the regulation of RNA synthesis and post-transcriptional control (51). TAM-ER complexes could certainly lead to alteration of these normal regulatory and processing steps. Part of the cytotoxicity of FUra may be due to its incorporation into RNA, with subsequent formation of "fraudulent" RNA, which may interfere with normal post-transcriptional processing (32). If at least 2 separate steps in the RNA processing pathway were blocked or altered by the respective affects of TAM and FUra, synergistic interaction of the 2 drugs may occur and would involve "sequential" inhibition (53). These two drugs by interferring with the normal production of mature RNA by the cell would have resulting adverse affects on normal cellular protein synthesis and metabolism.

Another model for the interaction of TAM and FUra involves a "complementary inhibition" model. Sartorelli described such a model for the interaction of agents which bind and inactivate macromolecules with drugs which alter biosynthetic pathways leading to formation of end-products which are either non-functional or only partially functional (54). The activities of TAM and FUra may easily be fit into such a

model,since TAM-ER complexes certainly interact with chromatin and or RNA molecules. As already mentioned, FUra may have ultimate effects on both DNA and RNA biosynthetic pathways.

The interaction of TAM and MF treatment demonstrates the value TAM may have as a synchronizing agent *in vitro*. Pretreatment of 47-DN with 10μM TAM followed by estrogen rescue and MF administration inhibits colony growth to <10% of control at the doses used, and the ICE value clearly shows synergistic interaction. This synergism was not observed when low dose TAM (0.01μM) was used in a similar manner. Synchronization of the tumor cells followed by release would be expected to enhance the toxicity of MF if MTX was given as a large proportion of the cells entered the S phase of the cell cycle together. MTX's ability to enhance FUra toxicity is dependent on the cells' synthesizing DNA and thus nucleotides, so that tetrahydrofolic acid pools may be depleted as a result of the MTX-block of dihydrofolate reductase (Fig. 4). When cells are not rescued from the inhibition produced by TAM, the combined effects of TAM and MF are antagonistic. This is to be expected, for TAM prevents DNA synthesis, which is necessary for maximum growth inhibition to occur upon administation of MF in the manner described above.

These findings have great importance in the design of rational schedules of treatment of breast cancer. Gewirtz and Cadman have shown the efficacy of MF in the treatment of advanced breast cancer. We now have information on effective ways of adding TAM, already widely used in the treatment of breast cancer, to this antimetabolite combination in the treatment of individuals whose tumors

have been shown to contain ER. TAM should not be given concurrently with MF, as in the study of Hubay et al. (34), but should rather be administered before MF treatment, in a manner similar to the protocol of Allegra et al. (35). TAM should not enhance the toxicity of MF to any cells of the hosts which do not contain ER. Because only a limited number of estrogen target organs exist in the body and do not include common sites of antimetabolite toxicity, the use of TAM should not significantly increase chemotherapeutic toxicity. The combination of its intrinsic growth inhibition of mammary tumors, its ability to enhance MF tumoricidal action, and its low host toxicity suggest that its appropriate inclusion in the chemo-hormonal therapy of breast cancer should yield protocols that give a highly favorably therapeutic index in the treatment of this significant disease.

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